PATENT
Docket No. 432722002600

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02-02-2001
Date

Julie Brazler

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Gregory R. Mundy, *et al.*

Serial No.: 09/113,947

Filing Date: 10 July 1998, RCE filed on 26 June, 2000

For: INHIBITORS OF PROTEASOMAL ACTIVITY [AND NF-☑B ACTIVITY] AND PRODUCTION FOR STIMULATING BONE GROWTH (AS AMENDED)

Examiner: Ralph Gitomer

Group Art Unit: 1623

**DECLARATION OF GREGORY R. MUNDY PURSUANT TO 37 C.F.R. §1.132**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Gregory R. Mundy, declare as follows:

1. I am one of the co-inventors of the subject matter claimed in the above-referenced application.

2. Other co-inventors and I have conducted experiments demonstrating that proteasome inhibitors promote osteoblast proliferation, differentiation of osteoblast precursors and bone growth. These experimental results are set forth in the following paragraphs 3-10 and in the attached Tables 1-3.

3. Osteoblast proliferation, differentiation of osteoblast precursors and bone growth can be shown by histologic observations of calvarial bone cultures, which show increased osteoblast numbers, new bone formation and morphologic maturation of osteoblasts (See detailed description of methods for determining new bone area and osteoblast numbers in Example 2 of the present specification at page 27, line 25 through page 29, line 3).

4. In the experiments described in Example 2 of the present specification, compound 59-0328 serves as a positive control. The two tested proteasome inhibitors are MG-132 and MG-115. As shown in Figure 1 (left column) of the present application as filed, both the positive control compound and the two tested proteasome inhibitors MG-132 and MG-115 promote new bone growth as evidenced by the new bone formation.

5. Promotion of osteoblast proliferation and new bone growth by structurally-unrelated proteasome inhibitors is demonstrated in the Table 1 submitted herewith. In this experiment, the negative control media contain no substance that promotes osteoblast proliferation and new bone growth. BMP2, a substance that is known to promote osteoblast proliferation and new bone growth, serves as a positive control. The four tested structurally-unrelated proteasome inhibitors are lactacystin, proteasome inhibitor 1 (PSI), epoxomicin and eponemycin. The osteoblast numbers treated with the negative control media are 104/0.3 mm bone (Table 1, far right column). The osteoblast numbers treated with the positive control BMP2 are 154/0.3 mm bone. The osteoblast numbers treated with lactacystin (as high as 160/0.3 mm bone), proteasome inhibitor 1 (as high as 179/0.3 mm bone), epoxomicin (as high as 173/0.3 mm bone) are equivalent to or higher than the osteoblast numbers treated with BMP2. The osteoblast numbers treated with eponemycin (as high as 114/0.3 mm bone), although lower than the osteoblast numbers treated with BMP2, is higher than the osteoblast numbers treated with the negative control media. Similarly, the new bone area treated with the negative control media is 3.1 $mm^2X10^{-3}$ (Table 1, second column from the right). The new bone area treated with BMP2 is 5.6 $mm^2X10^{-3}$. The new bone area treated with lactacystin (as high as 5.9 $mm^2X10^{-3}$), proteasome inhibitor 1 (as high as 8.2 $mm^2X10^{-3}$) and epoxomicin (as high as 6.8 $mm^2X10^{-3}$) is

equivalent to or higher than the new bone area treated with BMP2. The new bone area treated with eponemycin (as high as 3.5 $mm^2X10^{-3}$), although lower than the new bone area treated with BMP2, is higher than the new bone area treated with the negative control media.

6. Promotion of osteoblast proliferation and new bone growth by another proteasome inhibitor PSI-epoxide is also demonstrated in the Table 2 submitted herewith. The osteoblast numbers treated with the negative control media are 88/0.3 mm bone (Table 2b, middle column). The osteoblast numbers treated with PS1-epoxide are as high as 161/0.3 mm bone (Table 2b, middle column). Similarly, the new bone area treated with the negative control media is 2.8 $mm^2X10^{-3}$ (Table 2a, middle column). The new bone area treated with PS1-epoxide is as high as 5.64 $mm^2X10^{-3}$ (Table 2b, middle column).

7. Osteoblast differentiation can be analyzed by monitoring alkaline phosphatase in the media of these cultures because alkaline phosphatase is an established marker of osteoblast differentiation. Alkaline phosphatase activity in media of neonatal murine calvaria can be measured by the following method. Murine neonatal calvaria are cut and placed into BGJ media with 1 mg/ml bovine serum albumin containing a proteasome inhibitor, *e.g.*, PSI-epoxide, and incubated at 37°C in 5% $CO_2$ for 24 hours. Four half calvaria are used per treatment group. The media is then changed to fresh media again containing the proteasome inhibitor and incubated for a further 72 hours at which time the media is removed and assessed for alkaline phosphatase activity. Alkaline phosphatase activity is assessed for each calvarial culture by sampling 20 µl of media from each well and reacting it with 80 µl AMP buffer containing p-Nitrophenyl Phosphate, a specific substrate for this enzyme, and incubating for 17 min at 37°C reaction. The reaction is then stopped with 100 µl 0.5N NaOH and the subsequent p-Nitrophenol (yellow color) formation is read at 405 nm and is directly related to the amount of alkaline phosphatase activity present in the cultured media.

8. Promotion of differentiation of osteoblast precursors by eponemycin, PS1 and PS1-epoxide is demonstrated in the Table 3 submitted herewith. In the experiment using eponemycin (Table 3a, middle column), the alkaline phosphatase activity treated with a negative

control media is 0.022 unit. In contrast, the alkaline phosphatase activity treated with eponemycin is as high as 0.075 unit. Similarly, the alkaline phosphatase activity treated with PSI (Table 3b, middle column) and PSI-epoxide (Table 3c, middle column) are also higher than their corresponding negative controls.

9. In the experiments described in the above paragraphs 3-8, it has been observed that the proteasome inhibitors promote osteoblast proliferation, differentiation of osteoblast precursors and new bone growth without any discernible effect on bone resorption.

10. It is known in the art that inhibition of bone resorption does not mean enhancement of bone formation. In fact, reducing bone resorption usually inhibits bone formation because all of bone turnover is slowed. In this aspect, proteasome inhibitors are very different from inhibitors of bone resorption such as bisphosphonates, estrogen, raloxifene or calcitonin, in that the proteasome inhibitors promote osteoblast proliferation, differentiation of osteoblast precursors and new bone growth without any discernible effect on bone resorption.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Executed at San Antonio, Texas, on January 30, 2001.

Gregory R. Mundy

**Table 1.** Effects of structurally-unrelated proteasome inhibitors and the statin simvastatin at multiple concentrations on explants of neonatal murine calvarial bones cultures for 72 hours.

| Treatment | (μM) | New bone area (per $mm^2 \times 10^{-3}$) | Cells (per 0.3 mm bone) |
|---|---|---|---|
| Negative Control Media | | 3.1 ± 0.2 | 104 ± 5 |
| BMP2 (40 ng/ml) | | 5.6 ± 0.4* | 154 ± 5* |
| Lactacystin | 0.62 | 3.2 ± 0.5 | 105 ± 6 |
| | 1.25 | 4.7 ± 0.7 | 114 ± 7 |
| | 2.5 | 5.9 ± 0.9* | 160 ± 6* |
| | 5 | 5.4 ± 0.6 | 157 ± 6* |
| Proteasome inhibitor-1 | 0.006 | 4.3 ± 0.1 | 116 ± 4 |
| | 0.0125 | 5.5 ± 0.3* | 120 ± 6 |
| | 0.025 | 6.0 ± 0.9* | 132 ± 10* |
| | 0.05 | 8.0 ± 0.6* | 162 ± 15* |
| | 0.1 | 8.2 ± 1.2* | 179 ± 15* |
| Epoxomicin | 0.00062 | 2.8 ± 0.3 | 106 ± 8 |
| | 0.00125 | 2.8 ± 0.4 | 101 ± 7 |
| | 0.0025 | 4.0 ± 0.4 | 119 ± 5 |
| | 0.005 | 6.5 ± 0.8* | 162 ± 6* |
| | 0.01 | 6.8 ± 0.5* | 173 ± 6* |
| Eponemycin | 0.031 | 3.1 ± 0.5 | 98 ± 6 |
| | 0.062 | 2.9 ± 0.3 | 102 ± 8 |
| | 0.125 | 3.2 ± 0.4 | 105 ± 7 |
| | 0.25 | 3.4 ± 0.4 | 96 ± 5 |
| | 0.5 | 3.5 ± 0.8 | 114 ± 6 |
| Simvastatin | 0.062 | 5.3 ± 1.1 | 110 ± 9 |
| | 0.125 | 8.2 ± 0.7* | 135 ± 4* |
| | 0.25 | 14.5 ± 1.8* | 167 ± 17* |
| | 0.5 | 14.0 ± 1.1* | 190 ± 26* |

*Significantly greater than control media $p<0.05$

**Table 2**

**Table 2a.** New bone area in the media of murine neonatal calvaria treated with PSI-epoxide for 4 days

| Group | New Bone Area ($mm^2 \times 10^{-3}$) | SE |
|---|---|---|
| Negative Control | 2.80 | 0.37 |
| PSI –Epoxide 12.5nM | 3.46 | 0.35 |
| PSI –Epoxide 25nM | 4.27 | 0.33* |
| PSI –Epoxide 50nM | 5.64 | 0.31* |
| PSI –Epoxide 100nM | 5.56 | 0.20* |

**Table 2b.** Cell number on the surface of murine neonatal calvaria treated with PSI-epoxide for 4 days

| Group | Cell Number Per 0.3mm of bone | SE |
|---|---|---|
| Negative Control | 88 | 3 |
| PSI –Epoxide 12.5nM | 96 | 4 |
| PSI –Epoxide 25nM | 115 | 8* |
| PSI –Epoxide 50nM | 161 | 12* |
| PSI –Epoxide 100nM | 151 | 4* |

**Table. 3**

**Table 3a.** Relative alkaline phosphatase activity in the media of neonatal calvaria treated with Epoxomicin

| Group | Alkaline Phosphatase Activity (OD) | SE |
|---|---|---|
| Negative Control | 0.022 | 0.005 |
| Epoxomicin 0.62nM | 0.028 | 0.003 |
| Epoxomicin 1.25nM | 0.06 | 0.001* |
| Epoxomicin 2.5nM | 0.075 | 0.01* |
| Epoxomicin 5nM | 0.075 | 0.004* |
| Epoxomicin 10nM | 0.064 | 0.004* |

* - Significantly greater than Control group $p < 0.05$

**Table 3b.** Relative alkaline phosphatase activity in the media of neonatal calvaria treated with PSI

| Group | Alkaline Phosphatase Activity (OD) | SE |
|---|---|---|
| Negative Control | 0.039 | 0.005 |
| PSI 12.5nM | 0.039 | 0.004 |
| PSI 25nM | 0.072 | 0.01* |
| PSI 50nM | 0.096 | 0.01* |
| PSI 100nM | 0.108 | 0.01* |

* - Significantly greater than Control group $p < 0.05$

**Table 3c.** Relative alkaline phosphatase activity in the media of neonatal calvaria treated with PSI-epoxide

| Group | Alkaline Phosphatase Activity (OD) | SE |
|---|---|---|
| Negative Control | 0.040 | 0.011 |
| PSI –Epoxide 12.5nM | 0.044 | 0.003 |
| PSI –Epoxide 25nM | 0.074 | 0.006* |
| PSI –Epoxide 50nM | 0.121 | 0.013* |
| PSI –Epoxide 100nM | 0.107 | 0.005* |

* - Significantly greater than Control group $p < 0.05$